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A Manual of Rice Seed Health Testing

Edited by T.W. Mew and J.K. Misra

1994

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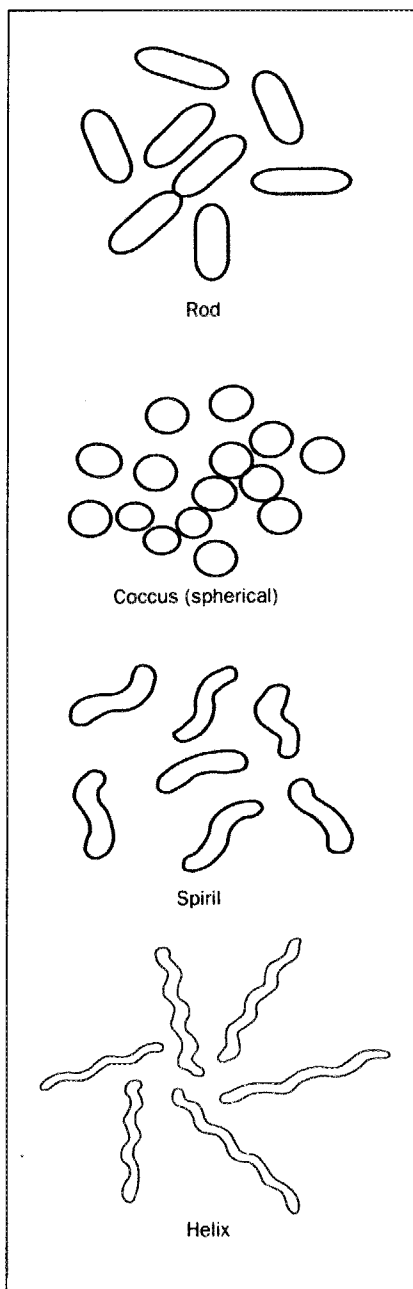
Bacteria

B. Cottyn, M.T. Cerez, and T.W. Mew

Bacteria (from bacterium, Greek, a small staff) are free-living unicellular organisms widely distributed in nature. They are prokaryotes—organisms with a single chromosome which is not enclosed in a nucleus. They possess a rigid multilayered cell wall that gives them a defined spherical-, rod-, helical-, or spiral-like shape (Fig. 7.1). Often the cells remain together in groups or clusters; for instance, it is very common for cocci or rods to occur in long chains. Bacteria reproduce asexually by binary fission. A bacterium growing on an agar surface forms a colony. Therefore, colony formation allows one to determine the number of bacteria in a culture. For instance, if 100 cells are plated, 100 colonies will appear. Bacteria can be grown in liquid or on solid growth media.

Bacterial rice pathogens

About 10 rice diseases are known to be caused by bacteria. The causal organisms are classified into three genera: *Erwinia*, *Pseudomonas*, and *Xanthomonas*. This manual discusses in detail only bacterial rice pathogens that occur commonly, are important for seed health, and are reported or suspected to be seedborne. Although a standardized, precise, and quick method of detecting, isolating, and characterizing bacterial rice pathogens has not yet been developed, some traditional methods described here can serve seed health testing purposes. Photographs of the species which concern this manual are given in Chapter 15.



7.1 Various Shapes of bacteria.

Detection tests are used to determine the presence of certain bacteria. If presence is indicated, the bacteria are then isolated. In fact, detection tests may serve as additional confirmation for identification tests, or as a first screening of unknown isolates to detect possible pathogens based on revealed symptoms or characteristics.

Erwinia species isolated from rice are commonly considered to be saprophytes although they may also act as opportunistic pathogens. Only two *Erwinia* spp. have been reported as rice pathogens: *E. herbicola* and *E. chrysanthemi*. *E. herbicola* was reported in Japan as the causal organism for palea browning, hence affecting rice grain quality. *E. chrysanthemi* causes foot rot which affects both the leaf sheath (causing sheath browning as in sheath rot) and the culm, and the root crown, resulting in severe decay at the foot of the rice plant. The disease has been reported in Japan (Goto 1979), Bangladesh, Korea, India, and the Philippines. However, this manual does not discuss the genus *Erwinia* further, since these pathogens have not been confirmed as seedborne. *E. chrysanthemi* is thought to be disseminated in irrigation water. The disseminating medium for *E. herbicola* remains a matter of controversy. It may or may not be seedborne.

This chapter will cover extraction and isolation, purification, identification, and detection of seedborne pathogenic *Pseudomonas* and *Xanthomonas* species. Recipes for all media, stains, and buffer preparations are supplied in Appendix 2.

Extraction and isolation

It is essential to be able to isolate one microorganism from all others and to maintain it in culture in a pure state. Only after a pure culture has been isolated can one proceed to study the organism's characteristics. (For schematic diagram of these processes, see Figure 7.2)

Pseudomonads

There are two main groups of phytopathogenic pseudomonads: the fluorescent group which produces a fluorescent pigment and the nonfluorescent group which does not.

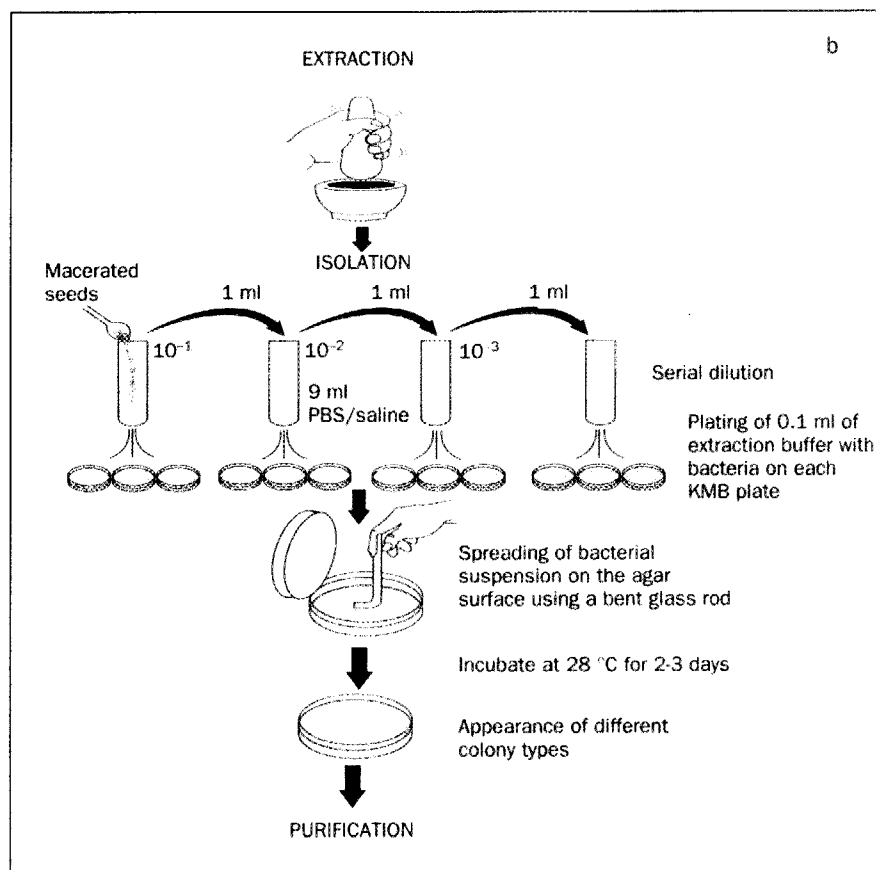
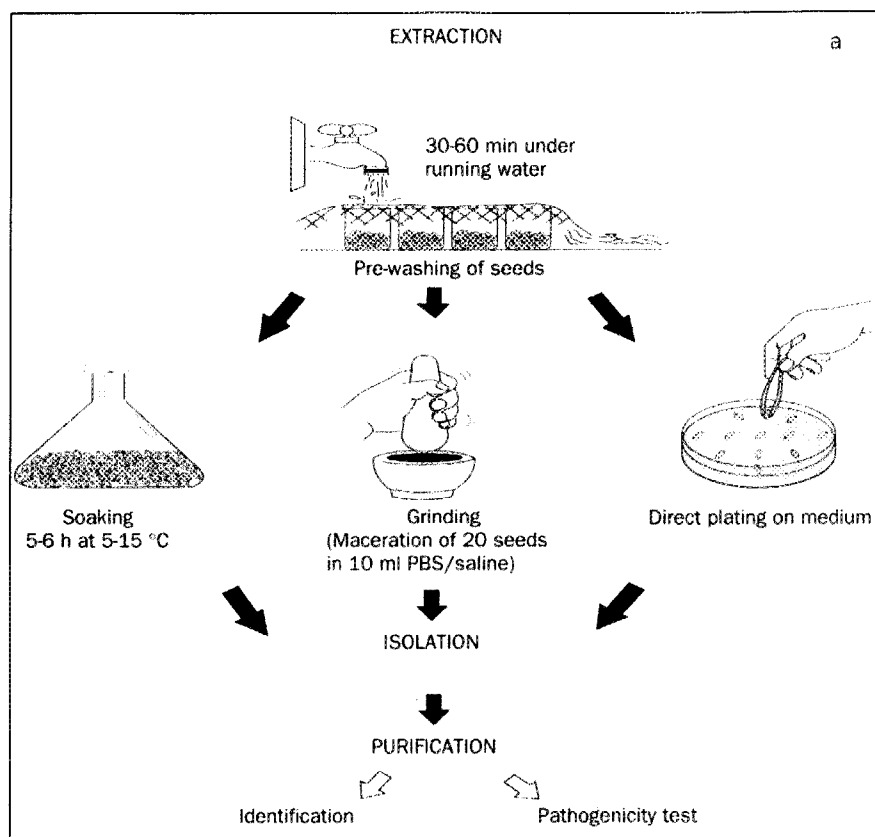
The most widely used medium for fluorescent pigment production is King's medium B (King et al 1954). Various other media and procedures for isolating *Pseudomonas* species can be found in the *Laboratory guide for identification of plant pathogenic bacteria* (Schaad 1990).

Seeds must often be prewashed to reduce debris and surface microflora, such as fungi, which otherwise will overgrow and interfere with the isolation of bacteria. To prewash, place beakers containing the different seed samples under running water for 30-60 min.

After prewashing, prepare sample extracts either by macerating the seeds or by soaking them for several hours at low temperature (5-15 °C) in phosphate buffer. The soaking method is usually used for large seed lots; for small-scale seed tests, grinding the seeds or plating them directly on medium is best. A sterile phosphate-buffered saline is the most commonly used extraction medium for both methods. Addition of detergents such as Tween 20

7.2a. Extraction of bacterial culture.

7.2b. Isolation of bacterial culture.



(0.001 %) to the extraction buffer can enhance the recovery of bacteria from the seeds, while addition of cycloheximide (0.01%) to the extraction buffer decreases fungal interference.

For the isolation step, 0.1 ml of undiluted extraction buffer and 10^{-1} , 10^{-2} , and 10^{-3} dilutions of the buffer are transferred onto 3 plates each of King's medium B. The 10^{-3} dilution is important because some seed lots can be contaminated with saprophytic or antagonistic bacteria which, when present in large numbers, might inhibit growth of some pathogenic pseudomonads. Bacterial suspension is spread on the surface of the agar, and the plates are then incubated.

Table 7.1 presents a grid of the morphological and biochemical characteristics that differentiate pathogenic pseudomonads.

7.2c. Purification of bacterial culture.

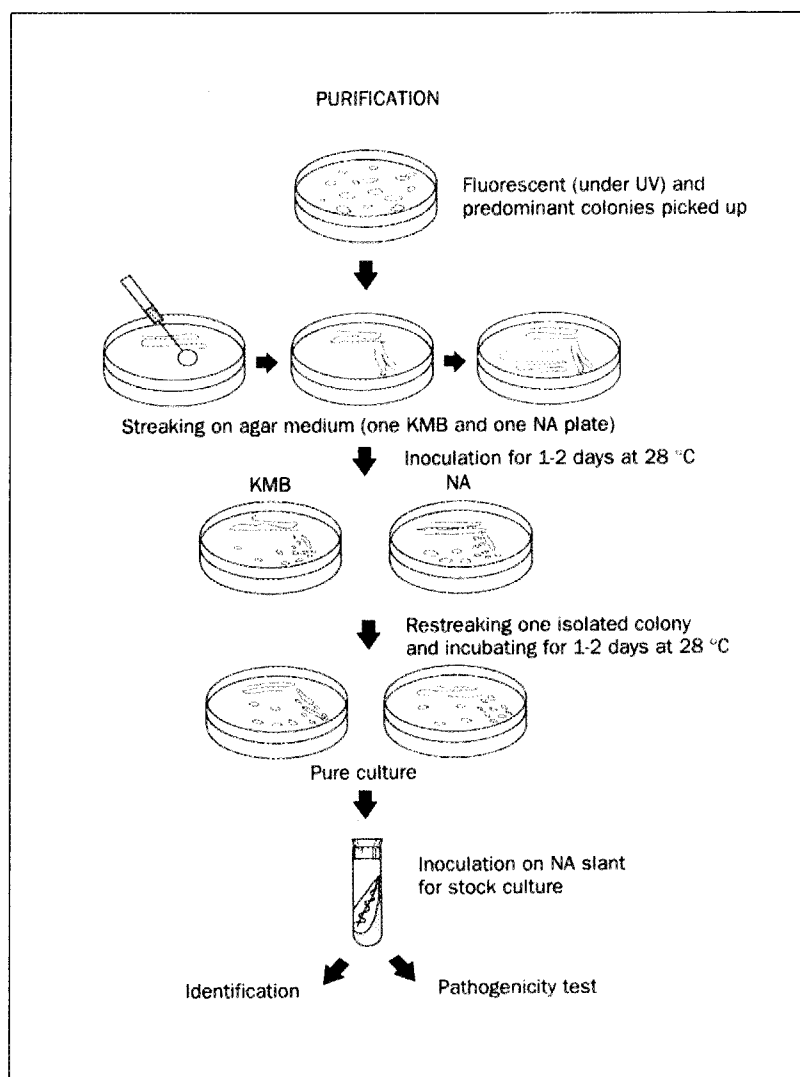


Table 7.1 Morphological and biochemical characteristics that differentiate pathogenic pseudomonads.

	<i>P. syringae</i> pv. <i>syringae</i>	<i>P. fuscovaginae</i>	<i>P. avenae</i>	<i>P. glumae</i>
Size	2.0-3.5 x 0.8-1.0 µm	0.5-0.8 x 2.0-3.5 µm	0.4-0.8 x 1.8-4.4 µm	0.5-0.7 x 1.5-2.5 µm
Fluorescence	+	+	+	-
Oxidase	-	+	+ ^{a,b}	-
Oxidation-fermentation (O-F) test	0	0	0	0
Nitrate reduction	-	-	+	+ ^{a,c}
2-ketogluconate production	-	-	-	-
ADH	-	+	-	-
Starch hydrolysis	-	+ ^e +/- ^f	+/-	+/- ^d -
Growth on				
Inositol	+	-	-	+
Trehalose		+	-	

^aZeigler and Alvarez 1989. ^bBradbury 1986, Bergey's manual 1986. ^cBergey's manual 1986. ^dBradbury 1986. ^eMiyajima et al 1983. ^fRott and Nottingham 1989.

GRINDING METHOD (FOR SMALL-SCALE SEED TESTING)

General: The following is one procedure for small-scale seed testing using the grinding method.

Procedure: 1. Thoroughly crush 20 seeds in 10 ml sterile phosphate buffer using either a sterile pestle and mortar or an electric mixer.

2. Leave suspension resting at room temperature for 2 h (25-28 °C). This allows bacteria to multiply to a more detectable level. (Pathogenic bacteria might represent only 1% of the total microflora of the seed.)

3. Vortex the suspension for 5 min and transfer 0.1 ml of undiluted and 10^{-1} , 10^{-2} , and 10^{-3} dilutions onto 3 plates each of King's medium B. Plate by using an L-shaped glass rod while spinning the plate, so that the liquid is evenly spread on the agar surface.

4. Incubate plates at 28 °C for 2-3 d.

5. Examine plates under UV light for fluorescent *Pseudomonas*. Pick up and restreak all fluorescent and predominant nonfluorescent colonies on both nutrient agar and King's medium B for purification.

Table 7.2. Morphological and biochemical characteristics that differentiate *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola*.

	<i>X. oryzae</i> pv. <i>oryzae</i>	<i>X. oryzae</i> pv. <i>oryzicola</i>
Size	0.5-0.8 X 1.3-2.2 µm	0.4-0.6 X 1.0-2.5 µm
Oxidase test	—	—
Oxidation-fermentation (O-F) test	0	0
Nitrate reduction	—	—
2-ketogluconate production	—	—
Starch hydrolysis ^a	±	+
Carbon source utilization		
Trehalose	+	+
Inositol	—	—
Alanine	—	+
0.001% CuNO ₃	+	—

^aBradbury (1986) registered Xoo as — and Xcola as +. Swings et al (1990) registered Xoo as +, + = positive, — = negative, 0 = oxidative.

Xanthomonads

Most species of the genus *Xanthomonas* are plant pathogens with a vast variety of plant hosts. Bacterial blight and bacterial leaf streak are two major rice diseases caused by *Xanthomonas* spp. Seed transmission of *X. oryzae* pv. *oryzicola* (Xcola) was confirmed by Goto et al (1988). Whether *X. oryzae* pv. *oryzae* (Xoo) is seed-transmitted remains a controversial issue despite intensive studies in both temperate and tropical countries (Mew 1989, Coto et al 1988).

Both pathogens are very closely related in many morphological, cultural, physiological, and biochemical characters. However, differentiation is possible by

- the symptoms they cause on rice,
- a few phenotypic features,
- polyacrylamide gel electrophoresis protein fingerprints,
- fatty acid profiles, and
- pathovar-specific monoclonal antibodies (Swings et al 1990).

Also, Xoo grows on media containing 0.001% CuNO₃ but not on media containing alanine as a sole carbon source, while Xcola grows on alanine but not on 0.001% CuNO₃ (Vera Cruz et al 1984).

Table 7.2 presents, in grid form, the morphological and biochemical characteristics that differentiate Xoo from Xcola.

DIRECT ISOLATION FROM SEEDS COLLECTED FROM NATURALLY INFECTED FIELDS

General: Infected seeds are ground into a fine powder and used for isolation. This is the same method described for the isolation of *Pseudomonas*, with a few modifications to make the method more suitable for the isolation of *Xanthomonas*.

Procedure: 1. Use peptone sucrose agar (PSA) instead of King's medium B as the isolation medium.

2. Incubate plates at 28 °C for 3-5 d.

3. Resultant yellow colonies which appear after 48 h and which are similar to Xoo and Xcola (see Figs. 15.5a-15.6a, pp. 92-93) are picked up and restreaked on PSA plates.

Note: Direct isolation of Xoo and Xcola from seed is quite difficult because these bacteria grow slowly on laboratory isolation media. This brings about the significant problem of major overgrowth by a wide variety of fast-growing contaminants. Moreover, many of these contaminants, such

as *Erwinia herbicola*, also have yellow colonies which hinder isolation. The recovery of Xoo and Xcola from seeds is very low, thus the isolation method from infected leaves (see below) is preferred for obtaining Xoo or Xcola.

DIRECT ISOLATION FROM LEAVES COLLECTED FROM THE FIELDS

Procedure: 1. *Selection from infected tissue.* Cut a 2- × 7-mm section from the advancing portion of the lesion. Sterilize leaf tissue in 70% alcohol for 15-30 s. Rinse the section in 2-3 changes of sterile distilled water in test tubes.

2. *Bacterial ooze.* Cut the disinfected tissue into tiny pieces with sterile scissors and place in 2-3 drops of 0.2 ml sterile physiological water (0.85% NaCl) in the isolation dish. Allow bacteria in these tissues to ooze for 5-10 min.

3. *Streaking on PSA plates.* Using a sterile wire loop (2 mm diam), obtain a loopful of bacterial suspension from the isolation dish and streak onto PSA plates.

4. *Incubation.* Keep streaked plates upside down at 28 °C for 72 h for Xcola and 96 h for Xoo.

Results: Observe plates daily after streaking. Saprophytic bacteria often appear after 24 h. Xoo colonies are visible only after 96 h, while Xcola appears after 72 h. When looking for Xoo, mark and reject bright yellow colonies that appear before 48 h. Select pale yellow and single mucoid colonies for purification.

Purification

General: After 2-3 d (for *Pseudomonas*) or 3-4 d (for *Xanthomonas*) of incubation, the growth on the isolation plates will exhibit a mixture of different colony types. However, before starting identification, the organisms must be isolated in pure cultures. Therefore the different colony types must be picked up and restreaked on nutrient agar and King's medium B (for *Pseudomonas*) or onto duplicate plates of PSA (for *Xanthomonas*).

Procedure: 1. Make streaks in three right angle directions; flame the loop after each directional streak.

2. Incubate plates again for 1-2 d (for *Pseudomonas*), 72 h (for Xcola), or 96 h (for Xoo) at 28 °C. In the first directional streaks, the colonies will be very close together, but in the later directional streaks, well-isolated colonies should be obtained.

3. From these plates, restreak one well-isolated colony on a fresh agar plate and incubate again. If all colonies are of similar size, shape, color, and texture, it is presumed that a pure culture has been obtained.

4. To preserve the culture in a pure state, transfer to a slant tube which can be stored at 4 °C for 2-4 wk, after which subculturing must be done again. Slants are made by autoclaving 5 ml of medium dispensed in glass tubes, preferably with screw caps. The medium is then solidified by cooling the tubes in an inclined position.

5. For *Xanthomonas*, select pale yellow distinct colonies from purified plates. Touch a single colony with a wire loop and transfer into slants of modified Wakimoto's medium by making a single vertical streak on the agar surface, then spreading the colony horizontally and evenly. For *Pseudomonas*, use nutrient agar slants.

6. Incubate slants at 28 °C for 24-48 h (for *Pseudomonas*) or 72 h (for *Xanthomonas*), then store at 4 °C.

Identification

Precise identification requires a combination of physiological and biochemical tests, selective media, and pathogenicity tests. Moreover, reliability of the test results should be confirmed by including a type strain in all identification tests.

Morphological characteristics

The usual procedure is to observe under a microscope the organism's size, shape, cell arrangement, and motility. Colony character and pigmentation are readily observed on the plates. A preliminary opinion of the kind of organism being dealt with can be determined from these characteristics. These tests form a necessary basis for identification, but do not complete the process, nor are their conclusions definitive.

CELL MORPHOLOGY

For observing the bacteria under the microscope, a 100X oil immersion objective and 8-12X eyepieces are normally used.

Pseudomonas are Gram-negative rods which are 0.5-1.0 µm by 1.5-4.0 µm and have one to many polar flagella. Cells occur singly or in pairs (occasionally in short chains).

Xanthomonas oryzae are Gram-negative straight rods with round ends which are 0.4-0.8 µm by 1.5-2.9 µm and have a single polar

flagellum. Cells occur singly, in pairs, or sometimes in chains.

Erwinia are Gram-negative straight rods, $0.5\text{--}1\text{ }\mu\text{m} \times 1\text{--}3\text{ }\mu\text{m}$, and possess 4–6 peritrichous flagella. Most cells occur singly. Endospores are not present in these genera.

MOTILITY

Bacteria are motile if they possess flagella. Two types of flagellar arrangements exist: polar and peritrichous. A bacterium with a single flagellum or a group of flagella at one or both poles of the cell is called polarly flagellated. Organisms with this flagellar arrangement move rapidly with a tumbling motion. Peritrichously flagellated bacteria have flagella attached around the surface of the cell. Such bacteria move more slowly, usually in a straight line, and rotate along the long axis. Motility is observed using a wet mount and preferably with a phase-contrast microscope. Only freshly grown cultures (grown overnight or for 1 d) should be examined since older bacterial cultures may be only weakly motile.

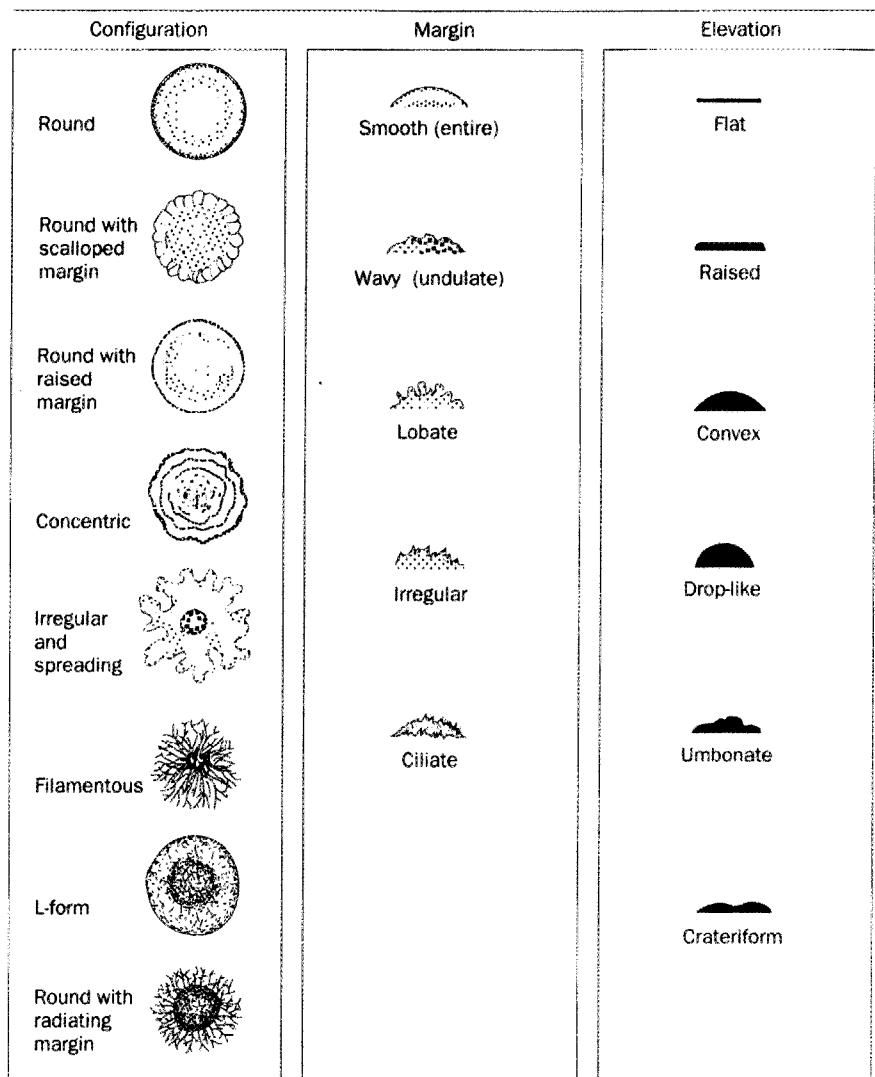
Procedure: 1. Place 1–2 drops of sterile physiological water (0.85% NaCl) on a clean glass slide.

2. Take a few bacteria by carefully dipping a sterile wire loop or sterile toothpick in a freshly grown colony and streaking them in the water on the glass slide.

3. Gently place a cover slip and observe motility under a 100X oil immersion objective.

COLONY CHARACTER

The form, elevation, and margin of bacterial colonies may help identify the type of bacterium (Fig. 7.3). In most cases, these properties lack diagnostic value unless the bacterium reveals a highly characteristic colony type under certain conditions—e.g., on certain selective media.



7.3 Morphology of bacterial colonies.

General colony appearance of the described *Pseudomonas* species on nutrient agar is as follows (see also Figs. 15.1a., 15.2a, 15.3a, and 15.4a).

■ *P. avenae* colonies are round, smooth, raised, chalk white, and glistening. Old colonies are sticky and adhere to the agar.

■ *P. fuscovaginae* colonies are round, smooth, raised, white to light brown, glistening, translucent, and 3-5 mm in diameter.

■ *P. glumae* colonies are round, smooth, raised, grayish white, and viscid.

■ *P. syringae* pv. *syringae* colonies are small, round, smooth, raised, whitish with a translucent margin which becomes undulate in older colonies.

Colony morphology of the described *Xanthomonas oryzae* pathovars on nutrient agar is as follows (see also Figs. 15.5a and 15.6a).

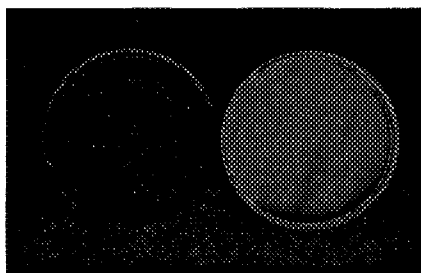
■ Xoo colonies are round, smooth, convex, butyrous, whitish yellow to straw yellow later, and opaque against transmitted light. The colonies appear as small dots on the 3rd or 4th d and reach 1-2 mm diameter on the 5th to 7th d—they grow slowly.

■ Xcola colonies are round, smooth, convex, viscid, whitish to pale yellow later with maturity. The colonies reach 1 mm diameter in 3 d—they grow much more rapidly than Xoo.

PIGMENTATION

The two main groups of phytopathogenic pseudomonads are the fluorescent group and the nonfluorescent group.

Some *Pseudomonas* species produce diffusible yellow-green pigments that are sometimes mistaken for fluorescent pigments. These can be distinguished by examining the cultures on solid media with ultraviolet light of short wavelength (254-360 nm) under which only the fluorescent pigments will fluoresce. The most widely used medium for fluorescent pigment production is King's medium B.



7.4 Fluorescence under UV light.
L = without fluorescent pigments,
R = with fluorescent pigments.

■ *P. fuscovaginae* and *P. syringae* pv. *syringae* belong to the fluorescent group and hence produce a fluorescent pigment on King's medium B (Fig. 7.4).

■ *P. avenae* and *P. glumae* belong to the nonfluorescent group. (*P. glumae* occasionally produces a diffusible yellow-greenish, nonfluorescent pigment.)

Yellow pigments are found in both Xoo and Xcola (as well as in *Erwinia herbicola* which is frequently isolated from rice). The yellow pigments from *Xanthomonas* have been given the common name *xanthomonadins* and are insoluble in water.

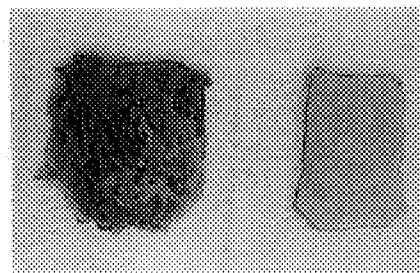
Biochemical characteristics

GRAM STAINING

General: This stain is essential for differentiating bacteria in two broad groups: Gram-positive and Gram-negative.

Gram-staining characteristics are related to structural and chemical properties of the cell wall. These characteristics are basic to the initial identification of plant pathogenic bacteria.

Procedure: 1. On a clean slide, thinly spread a bacterial film (a few bacteria spread in a drop of sterile distilled water on the glass slide). Dry in air for a few minutes. Then lightly flame the underside of the slide to fix the bacteria to it.

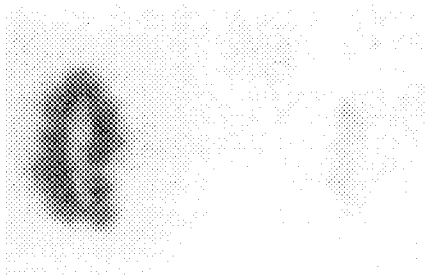


7.5 Gram staining.
L = color of Gram-positive reaction,
R = color of Gram-negative reaction.

2. Flood the smear with crystal violet solution for 1 min.
 3. Wash in tap water a few seconds. Drain off excess water.
 4. Flood the smear with iodine solution for 1 min.
 5. Wash in tap water a few seconds.
 6. Decolorize (about 30 s) with solvent (e.g., acetone-alcohol decolorizer) until the solvent flows colorless from the slide.
 7. Rinse in tap water for about 2 s.
 8. Counterstain for 3 min with safranin solution.
 9. Wash briefly in tap water. Dry in the air and examine under the microscope.
- Results: Gram-positive bacteria appear purple to blue-black. Gram-negative bacteria appear red (Fig. 7.5).

OXIDASE TEST

General: This test determines the presence of cytochrome c (oxidase enzymes of the respiratory chain) and is positive only for bacteria containing cytochrome c as a respiratory enzyme. Oxidase-positive organisms



are usually either aerobes or facultative anaerobes.

Obligate anaerobic organisms lack oxidase activity since they are unable to live in the presence of atmospheric oxygen and do not possess a cytochrome oxidase system.

- Procedure:**
1. Place a piece of Whatman No. 1 filter paper in a petri dish.
 2. To the paper, add some freshly prepared Kovacs' oxidase reagent (1% aqueous solution of tetramethyl-p-phenylenediamine dihydrochloride).
 3. With a platinum wire inoculating needle, smear a colony onto the reagent-impregnated paper in a line 2-3 cm long.

Results: A purple color (positive) occurs within 5-10 s if the organism is oxidase-positive—an anaerobe or a facultative anaerobe (Fig. 7.6). *P. avenae* may be positive or negative, *P. fuscovaginae* are oxidase-positive, and *P. syringae* pv. *syringae* and *P. glumae* are negative.

7.6 Oxidase test reaction. L = color of an oxidase positive reaction, R = oxidase negative reaction: no color around the streak of yellow pigmented bacteria.

OXIDATION-FERMENTATION (O-F)—THE TWO TUBES TEST

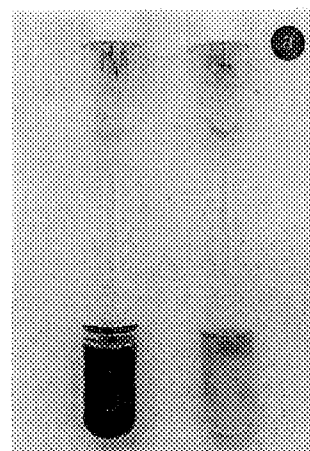
General: The Hugh and Leifson O-F basal medium is used to differentiate fermentative from oxidative metabolism of carbohydrates. Glucose is commonly employed in the O-F basal medium; however, there are times when an organism being tested is unable to metabolize glucose, yet can utilize other carbohydrates.

Fermentation is an

- anaerobic process and bacteria that ferment carbohydrates are usually facultative anaerobes—they can grow and reproduce under either aerobic or anaerobic conditions.

Oxidation of glucose is an aerobic process and bacteria that oxidize carbohydrates are usually strictly aerobic.

- Procedure:**
1. For each organism tested, inoculate a pair of O-F tubes with a small loop containing cells from a young culture.
 2. Set up control sets—one inoculated set with no carbohydrate added and one uninoculated set with carbohydrate. Include a positive control by inoculating a well-known fermentative organism such as *Escherichia coli*.



7.7 Oxidation-fermentation test.
a. Results of oxidation. b. Results of fermentation.

3. To detect fermentation, cover the medium in one tube with sterile mineral oil to a depth of 1-2 cm to exclude all oxygen.
4. Incubate at 28 °C for 48 h or longer.

Results: Observe for production of acid. If present, acid will cause a yellow discoloration in the medium because of pH change.

Oxidative organisms, such as *Pseudomonas* and *Xanthomonas*, produce acid reactions (resulting from the breakdown of glucose) in the uncovered medium only.

Lack of acid production in both tubes indicates that the organism is either unable to catabolize the glucose or the medium is unsuitable for growth of the organism.

Yellow color in the uncovered tube alone indicates oxidation (Fig. 7.7a); yellow color in both tubes indicates fermentation (Fig. 7.7b), and no color change in both tubes or a blue color (denoting alkaline reaction) indicates that neither oxidation nor fermentation took place.

Note: Of the organisms under discussion, *Erwinia* is the only fermentative one; both *Pseudomonas* and *Xanthomonas* are oxidizers.

NITRATE REDUCTION TEST

General: This test determines the ability of an organism to reduce nitrate to nitrite, which in turn can be further reduced to free nitrogen gas. The process is an anaerobic respiration whereby an organism is able to derive its oxygen from nitrate. The reduction of nitrate is denoted by the development of color, as a colored compound is formed by the reaction of nitrite with the two added reagents (sulfanilic acid and dimethyl- α -naphthylamine).

Procedure A:

1. Prepare potassium nitrate agar (semisolid) and place 5 ml portions in each tube (one tube per isolate).
2. Autoclave tubes containing the medium and cool before use or refrigerate for storage.
3. Stab-inoculate tubes using a wire loop with an 18-24-h-old pure colony grown from a nutrient agar plate.



7.8 Nitrate reduction. L = red color shows nitrate reduction, R = no color change means no nitrate has been reduced.

4. Set up control tubes.

Negative control: Inoculate uninoculated control tube and test with reagents in conjunction with inoculated tubes to determine if the initial medium is nitrite-free.

Positive control: Inoculate one tube with a known nitrate positive organism (e.g., *Escherichia coli*) and test to determine if the medium and reagents are functioning properly.

5. Incubate the tubes at 28 °C for 48 h.
6. Add nitrate reagents to an incubated nitrate agar tube: 5 drops of reagent A (0.6% dimethyl- α -naphthylamine) together with 5 drops of reagent B (0.8% sulfanilic acid) both dissolved in 30% 5 N acetic acid.

Results A: 1. If a pink to deep red color develops within 1-2 min, the test is positive (Fig. 7.8). The organism reduced nitrate to nitrite. The test is completed. *P. avenae* reduces nitrate; *P. glumae* may or may not

reduce nitrate; *P. syringae*, *P. fuscovaginae*, and *Xoo* and *Xcola* do not reduce nitrate.

2. If no color develops, no nitrite is present in the medium. The result is negative, or the nitrate has not been reduced, or the nitrite has been further reduced to free nitrogen. Continue to procedure B to test for presence of unreduced nitrate.

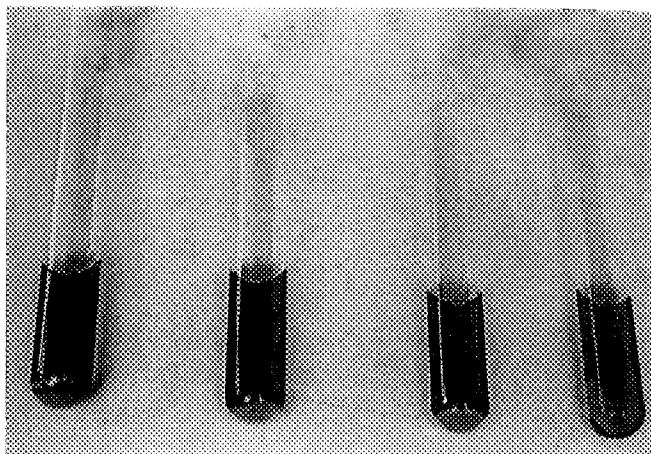
Procedure 8: Zinc reduction. To the test tubes which did not develop color, add a pinch (approximately 20 mg) of nitrate-nitrite-free zinc dust.

Results B: 1. Positive if no color develops—nitrate is absent, and the organism reduced nitrate to nitrite and then further reduced nitrite to free nitrogen or other end products.
2. Negative if deep red color occurs within 5-10 min—the zinc reduced the nitrate, which was still present in the test tube, to nitrite. The organism did not reduce the nitrate.

PRODUCTION OF 2-KETOGLUCONATE

General: The ability of an organism to oxidize gluconic acid (potassium gluconate) as its sole carbon source can easily be determined by the formation of the reducing compound 2-ketogluconate. Since 2-ketogluconate is a reducing agent, it can be detected by adding cupric ions, which are present as copper sulfate in Benedict's reagent. The originally blue cupric ions change to a yellow or orange-red precipitate of cuprous oxide when reduced.

Procedure: 1. Prepare gluconate peptone broth and dispense 2.0 ml per tube (one tube per isolate).



7.9 Production of 2-ketogluconate. The yellow precipitates in the three tubes on the left show a positive reaction. The clear blue solution with no precipitate in the tube on the right indicates a negative reaction.

2. Include an uninoculated tube in the test as a negative control.
3. Autoclave the tubes together with the broth and let them cool before use.
4. Inoculate the broth heavily from an 18-24 h pure culture grown on nutrient agar.
5. Incubate the inoculated tubes at 28 °C for 48 h.
6. Add 1.0 ml of Benedict's reagent directly to the incubated gluconate tube. Mix the solution well and place the tube in a boiling water bath for 10 min.

Results:

1. Positive—a yellow to orange-red precipitate indicates the presence of 2-ketogluconate as reducing substance (Fig. 7.9).
2. Negative—if there is no change, there is no precipitate. There is no reducing substance (2-ketogluconate) produced.
3. The uninoculated control tube should show negative results.

ARGININE DEHYDROLASE TEST

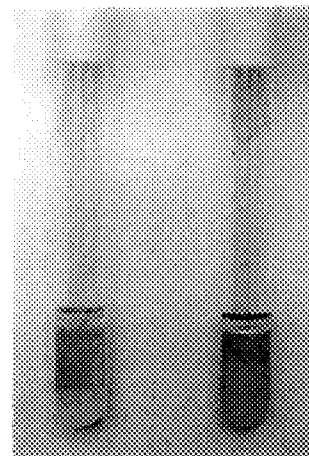
General: This test measures the ability of an organism to catabolize arginine. The arginine dehydrolase enzyme system couples

arginine degradation to adenosine triphosphate (ATP) generation, thus permitting the organism to grow under anaerobic conditions. The bacterium to be studied is cultivated under anaerobic conditions by overlaying the surface of the medium with either paraffin or mineral oil. L-arginine breakdown involves: two enzymes: arginine desmidase which degrades arginine to citrulline + NH_3 , and citrulline ureidase which converts citrulline to ornithine + CO_2 + NH_3 . The process can be monitored by including a pH indicator (phenol red) since the pH of the medium rises (to alkalinity) as NH_3 is produced.

Procedure: 1. Prepare the arginine medium and dispense 3 ml volumes in test tubes (one tube per isolate).

Include a control tube without arginine for each organism to be tested and an uninoculated tube with arginine.

2. Sterilize the tubes by autoclaving for 15 min and cool before use, or refrigerate for storage.
3. Stab-inoculate into the medium a freshly grown test organism (a culture grown



7.10 Arginine dehydrolase test. L = positive, R = negative for production of NH_3 .

for 18-24 h on nutrient broth).

4. Overlay all tubes with 2 ml of either sterile paraffin or sterile mineral oil to create an anaerobic condition.
5. Incubate the tubes for 3 d at 28 °C.

Results:

1. Positive (alkaline) if the medium turns red, due to production of NH_3 . Only *P. fuscovaginae* produces NH_3 (Fig. 7.10).
2. Negative if there is no color change, indicating no degradation of arginine (Fig. 7.10).
3. The inoculated control tube without arginine and the uninoculated one with arginine should remain yellow (the initial color).

STARCH HYDROLYSIS TEST

General: The starch hydrolysis test assesses an organism's ability to hydrolyze starch by enzymatic activity. Starch is a homopolysaccharide composed of many α -D-glucose units. The basic structure of starch is a mixture of two polyglucose molecules:

linear amylose (10-20%) and branched amylopectin (80-90%).

Starch hydrolysis occurs by enzymatic action of α -amylase (also called endoamylase). This digestive enzyme attacks the interior of polysaccharide chains. Amylose is split completely into maltose and glucose units, while complete breakdown of amylopectin requires the additional presence of another enzyme, glucosidase. Partially digested starch molecules are called dextrans. The more the starch is hydrolyzed, the smaller the dextrans become.

Starch hydrolysis can be followed using an iodine reagent. As the enzymatic reaction proceeds (i.e., the large polysaccharides are split into smaller units), the color produced by iodine gradually changes from blue to purple to red-brown (partial hydrolysis) to no color (complete hydrolysis).

Procedure: 1. To assure that the reagents are effective, they should be tested on known starch-hydrolyzing bacteria (e.g., *Bacillus subtilis*) and nonstarch-hydrolyzing bacteria (e.g., *E. coli*).
2. Prepare starch medium as described in Appendix 2.
3. Inoculate the starch agar plates by streaking with an 18-24 h pure culture from nutrient agar.
4. Incubate the inoculated plates at 28 °C for 5 d.
5. Flood the incubated plates directly with the reagent (aqueous Lugol's iodine) and interpret the reaction immediately.

Results: *P. syringae* does not hydrolyze starch; *Xcola*

does; *P. avenae*, *P. fuscovaginae*, *P. glumae*, and *Xoo* may or may not hydrolyze starch.

1. Positive if the medium is purple-blue with slight yellow or colorless zones (hydrolysis zones) around or under the bacterial growth. In this case, starch is completely hydrolyzed by the bacteria.
2. Negative if the medium and the area around the bacterial growth are purple blue. This indicates that starch is still present, and no hydrolysis occurred. The bacteria lack the ability to hydrolyze starch.
3. Partial hydrolysis has occurred if reddish-brown zones occur around growth, indicating the presence of dextrans. The bacteria are not able to complete starch hydrolysis.

CARBON SOURCE UTILIZATION

General: This procedure tests the ability of an organism to utilize certain sugars (e.g., trehalose and inositol, Tables 7.1 and 7.2 as a sole source of carbon. Utilization is demonstrated by the presence of growth.

The medium for the carbon source utilization tests comprises the inorganic minerals for growth and the sugar to be tested.

Procedure: 1. Prepare the Ayers et al mineral salts medium and sterilize by autoclaving at 121 °C for 15 min.
2. The carbon source (sugar) to be tested is filter-sterilized and added at 0.5% (wt/vol) final concentration to the autoclaved and cooled (45 °C) Ayers et al mineral salts medium. Prepare control plates without

sugar.

3. Pour the medium in petri dishes (one plate per isolate) and let them solidify in a sterile flow-bench.
4. Streak freshly grown bacteria onto the medium and incubate at 28 °C for 3, 7, and 14 d.

Results: Record presence or absence of growth as an indication of the organism's ability to utilize the tested sugar. Compare growth in plates containing no added sugar.

P. syringae and *P. glumae* grow on inositol and not on trehalose; *P. fuscovaginae* grows only on trehalose; *P. avenae* does not grow on either.

Both *Xoo* and *Xcola* grow on trehalose but not on inositol; however, *Xoo* will grow on CuNO_3 but not on alanine, while *Xcola* will grow on alanine but not on CuNO_3 .

Detection

Pathogenicity test

Pathogenicity tests have not been standardized. There are numerous applicable inoculation methods, including:

- inoculation by blade, scissors, or syringe;
- spraying the plants with a bacterial suspension;
- vacuum infiltration of bacteria into plant tissue; and
- wound inoculation by rubbing plant parts with a bacterial suspension.

All of these techniques have value, depending on the nature of the disease and purpose of the experiment.

Some principles stated in the *Laboratory guide for identification of plant pathogenic bacteria* (Schaad 1990), when making pathogenicity tests are quoted below:

1. Grow pathogen-free plants under conditions most favorable for their growth and which most closely approximate the conditions for disease development in the field.

2. Select an inoculation technique which most closely simulates the nature method of inoculation or infection.

3. Use relatively low dosages of bacteria in inoculation (10^3 to 10^5 colony-forming units(cfu)/ml) when spraying or infiltrating plants.

4. Symptoms obtained from plant inoculation should closely resemble those that occur in the field.

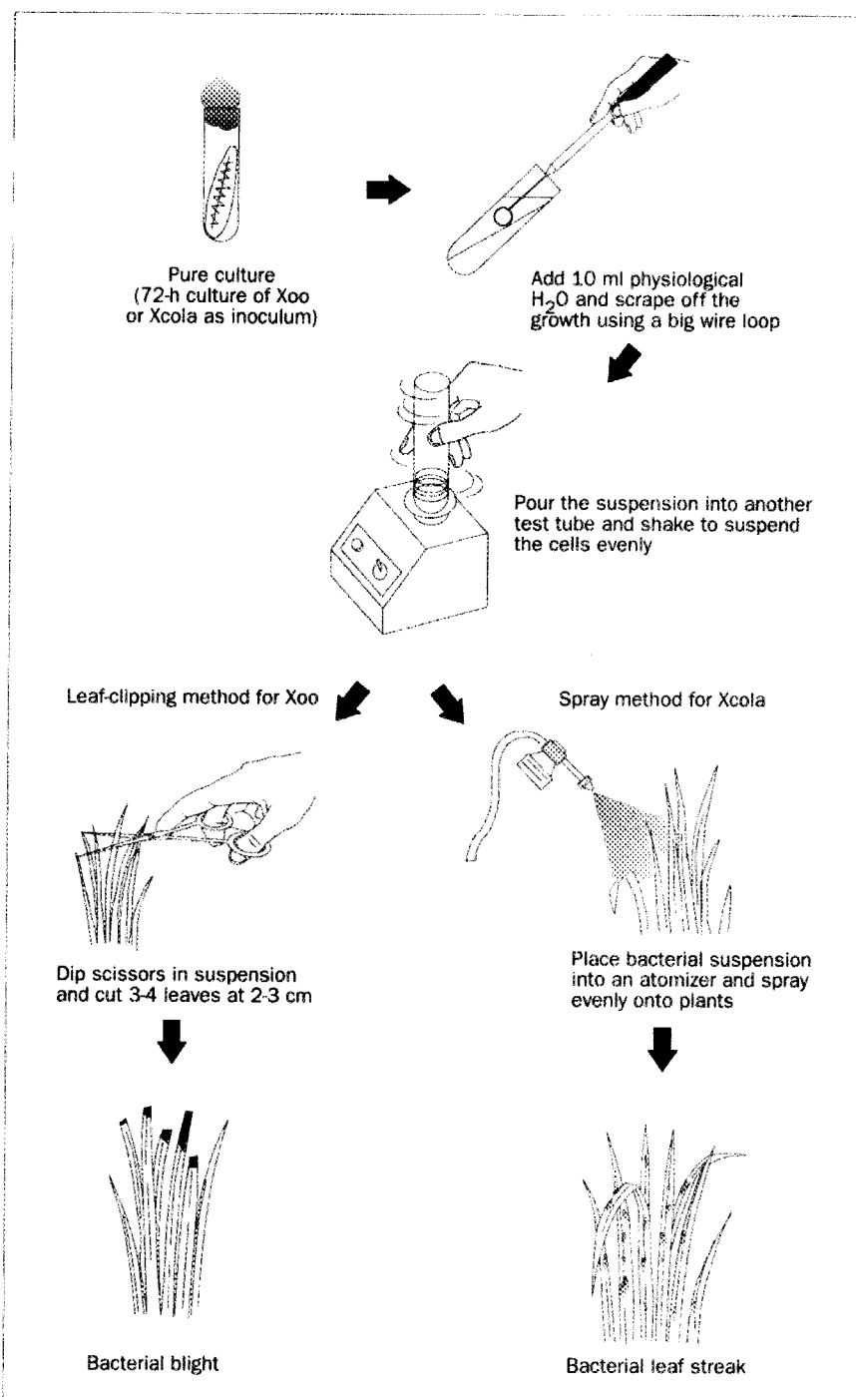
PSEUDOMONAS PATHOGENICITY TEST

General: The following is one method used at IRRI for detecting pathogenic *Pseudomonas* associated with rice seed. After isolating and purifying suspected bacteria from diseased seed samples, a pathogenicity test on seedlings is performed in the greenhouse.

Procedure: 1. *Plant preparation.* To clean the seeds, wash in 70% ethanol for 30 s and rinse three times in sterile distilled water. To germinate, place seeds on moistened filter paper in petri dishes and put in a growth chamber. After 1 wk, transplant seedlings to soil in plastic trays and put in a greenhouse.

2. *Preparation of inocula.* Inoculate the nutrient broth with seed isolates collected from nutrient agar slants and grow overnight. These nutrient broth cultures will be used to inoculate the seedlings.

3. *Inoculation by injection.* Inoculate 3- to 4-wk-old seedlings by injecting 0.3 ml bacterial suspension into the sheath. Inoculate 3 seedlings per isolate. Inoculate controls by injecting nutrient broth only.



7.11 Pathogenicity test for Xoo and Xcola on mature plants in the greenhouse.

Results: Disease symptoms are recorded 1 wk after inoculation. Carefully observe and note typical symptoms and the nature of the lesions. Due to the nature of the disease symptoms, presumptive evidence concerning the causal organism can sometimes be made. Nevertheless, the organism should be reisolated from any resulting lesions, subcultured, and identified (Koch's postulate).

Notes: Environmental conditions (temperature, relative humidity, presence of other interfering organisms, etc.) can have an important impact on disease development.

Symptoms caused by the *Pseudomonas* spp. discussed in this manual may be very similar, thus preventing reliable diagnosis by symptomatology alone. Nevertheless, pathogenicity testing is a useful tool to study disease symptoms and to detect the possible causal organism. Once an organism is suspected to be pathogenic, further characterization and identification must be made.

XANTHOMONAS PATHOGENICITY TEST (FOR MATURE PLANTS IN A GREENHOUSE)

General: A pathogenicity test is one of the most reliable methods for confirming bacterial identity from infected seeds or diseased seedlings. Some rice varieties are resistant to bacterial blight and bacterial leaf streak, thus it is important to use a susceptible variety when doing pathogenicity tests for these diseases.

Xoo infects the vascular tissues of leaves; thus all methods of artificial inoculation used for bacterial

blight, such as clipping and pin-prick methods, are based on wounding the leaves and simultaneously introducing the bacteria into the vascular system. Both the pin-prick and clipping methods are effective but the latter is faster (Fig. 7.11).

Natural Xcola infection is believed to occur through the stomata of the rice leaves. Therefore, the spraying method has been proven effective for bacterial leaf streak inoculation. Spraying is convenient and gives more reliable results than methods that wound the leaves (Fig. 7.11).

Procedure A: Preparation of inoculum

1. Grow a pure bacterial culture in a slant of modified Wakimoto's medium for 72 h.
2. Pour 10 ml sterile physiological water (0.85% NaCl) into the culture slant and scrape the bacterial mass off with a sterile wire loop.
3. Suspend the cells evenly in a mixer. The resulting suspension gives approximately 10^8 - 10^9 cfu/ml.

Procedure B: Inoculation of plants

1. *Inoculation for bacterial blight* (Xoo): Using the leaf-clipping method, inoculate susceptible varieties (e.g., IR24 or IR8) when they are 35-40 d old. Cut only fully expanded leaves, 2-3 cm from the tip, with a pair of sterile scissors dipped in bacterial suspension. To assure inoculation, clip only 3-4 leaves before dipping the scissors again in the inoculum. Label plants with date and isolate code, and keep in a greenhouse until scoring date.
2. *Inoculation for bacterial leaf streak* (Xcola): Place bacterial suspension, together

with a drop of sticker (Tween 20), into an atomizer and spray evenly onto IR24 or IR50 plants. After spraying, plants should appear moistened, but the suspension should not be dripping from them. Label plants as above and keep in a greenhouse.

Procedure C: Scoring. Xoo: In practice, disease symptoms are recorded 2 wk after inoculation in case of screening for varietal resistance. Since this procedure aims to determine the identity of the organism, 1 wk after inoculation may be enough time to observe typical bacterial blight symptoms—wavy, elongated, water-soaked lesions.

Xcola: Bacterial leaf streak lesions appear about 10 d after inoculation (Fig. 15.8b,c, and d).

If no characteristic symptoms are produced, the isolates may be considered as saprophytes.

Reisolation and confirmation of virulence from the lesions are necessary to confirm identity of the pathogen.

Semiselective media

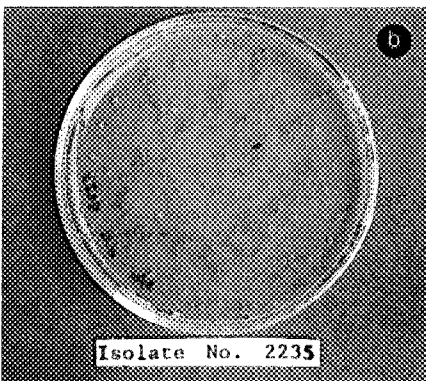
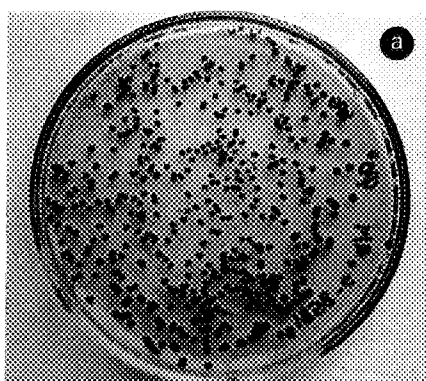
PSEUDOMONAS GLUMAE — TYPICAL

COLONY FORMATION

General: A selective medium (S-PG) was developed by Tsushima et al (1986) and was used by Mogi (1988) to detect *P. glumae* in rice seed. On the S-PG medium, *P. glumae* forms two types of colonies (Fig. 7.12), depending on the isolate:

■ Type A (Fig. 7.12a) colonies are round, smooth, convex, and reddish brown.

■ Type B (Fig. 7.12b) colonies are round, smooth, convex, and opalescent purple.



7.12 a. *P. glumae* type A colony on S-PG medium.
b. *P. glumae* type B colony on S-PG medium.

Other pseudomonads (fluorescent and nonfluorescent) may grow on this medium, but only *P. avenae* is reported to produce colonies similar to type B.

- Procedure: 1. Prepare the S-PG medium.
2. Make serial dilutions of 10^{-2} , 10^{-4} , and 10^{-6} in sterile physiological water (0.85% NaCl in distilled water) from a culture grown for 12 h in nutrient broth.
3. Pipette 0.1 ml of the serial dilutions on 2 plates of S-PG medium. Spread the bacteria out over the medium with an L-shaped glass rod while rotating the plate.
4. Incubate the plates at 28 °C for 3 d.

5. Check the plates for colony formation typical of *P. glumae*.

Notes: At IRRI, *Xanthomonas* did not grow on S-PG, but all the nonfluorescent pseudomonads tested grew well on S-PG and showed type B colonies. Although their identity is not yet confirmed, the colonies might be *P. avenae* (found to produce colonies on S-PG medium similar to type B of *P. glumae*). Type A colonies are more conclusively characteristic.

PSEUDOMONAS GLUMAE—CALCIUM OXALATE CRYSTAL FORMATION

Matsuda et al (1988) observed that when *P. glumae* was grown on potato peptone glucose agar (PPGA), a selected medium for *P. glumae* supplemented with 0.1% CaCl_2 , characteristic crystals were produced in the colony. This appears to be useful for rapid detection of *P. glumae*. However, at IRRI we were unable to observe any crystals.

PSEUDOMONAS FUSCOVAGINAE—MIYAJIMA'S SELECTIVE MEDIUM

General: Miyajima (1989) developed a selective medium to detect *P. fuscovaginae*. After 4-5 d, *P. fuscovaginae* grown on this medium produces round, smooth, raised, translucent, beige

or cream colonies, some of which have green pigments in the center (Fig. 7.13). The green pigments fade after 8 d or more.

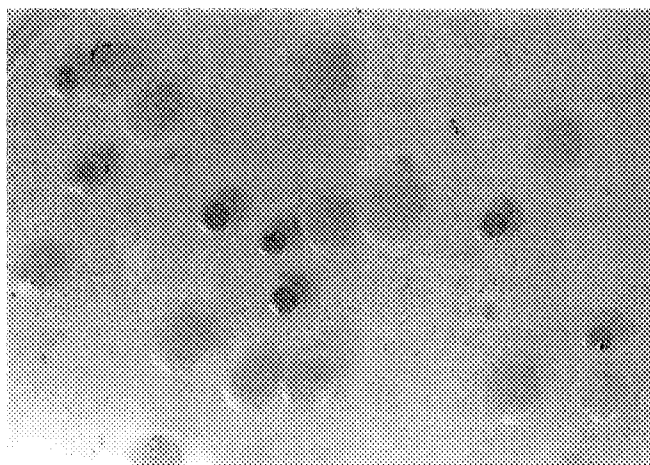
- Procedure: 1. Prepare Miyajima's medium.
2. Make serial dilutions of 10^{-2} , 10^{-4} , and 10^{-6} in sterile physiological water (0.85% NaCl in distilled water) from a culture of *P. fuscovaginae* grown for 12 h in nutrient broth.
3. Pipette 0.1 ml of the serial dilutions on the medium. Spread the bacteria over the medium with an L-shaped glass rod while rotating the plate.
4. Incubate the plates at 28 °C for 5 d.

Results: Check the plates for colonies which show the green pigmented spot in the center, typical of *P.*

fuscovaginae (Fig. 15.2a). Notes: Suspected *P. fuscovaginae* strains grown on this medium showed two different colony types in one plate.

Type 1: round, smooth, raised, translucent, light brown colonies with a green spot in the center.

Type 2: round, smooth, raised, opaque, cream-colored colonies without green pigments, and somewhat larger diameter.



7.13 *Pseudomonas fuscovaginae* on Miyajima's medium showing beige or cream-colored colonies. Note some colonies with green pigment at the center.

A MODIFIED BLOTTER TEST TO DETECT SEEDBORNE *P. AVENAE* (SHAKYA AND CHUNG 1983)

General: Bacterial stripe caused by *P. avenae* has been shown to result from naturally infected seeds. Shakya and Chung observed that symptom development increased when nitrogen was added. They developed a method for detecting *P. avenae* in which rice seeds are plated on filter paper moistened with 230 ppm nitrogen (urea) solution.

The test is described for 100 seeds (4 replicates of 25 seeds each).

- Procedure:**
1. Plate 25 seeds in petri dishes (9 cm diam) on 3 layers of filter paper moistened with 230 ppm of urea solution.
 2. Incubate plates at 27-30 °C and 12-h daylight cycles.
 3. After 3 d, remove lids so that seedling growth is not hindered. Flood seeds again with urea solution. Keep plates in a high-humidity tent (e.g., in a polyethylene bag) to prevent seeds from drying out.
 4. Open polyethylene bag periodically to allow air circulation.
 5. During the first week, add nitrogen solution 2-3 times.
 6. Starting from the second week, add only sterile water to keep filter papers well-moistened.
 7. Record symptoms after 12-14 d of incubation.

Results: Distinct brown stripes on the coleoptile, leaf sheath, and leaf blade are characteristic symptoms produced by *P. avenae* (Fig. 15.1b and c).

Phage techniques to detect Xoo

Fang et al (1982) stated that the existence of a Xoo species-specific phage in rice seeds was related to disease occurrence. Hence, isolation of the Xoo bacteriophage from infected materials indirectly indicates the presence of the pathogen.

Although phage techniques are an indirect method of detecting Xoo, they have proven quite sensitive and can detect as few as 10^2 cfu/ml of a pure Xoo culture (Katznelson and Sutton 1951). However, it is difficult to detect Xoo populations below 10^4 cfu/ml from samples which have high concentrations of saprophytic microorganisms (Goto 1971). Also it was reported by IRRI that the phages seem to survive much longer than do bacterial cells, particularly at higher temperatures (IRRI 1969). Phage techniques are also used to assay the disinfecting effect of various seed treatments for controlling the bacterial blight disease.

For an overview of the presently identified phage strains in different regions, refer to *Rice diseases* by Ou (1985).

Phage techniques can be applied in two ways to detect the presence of Xoo:

- by demonstrating the presence of the bacteriophage of Xoo in diseased leaves, infected seeds, or in ricefield water; or
- by using a Xoo-specific phage to identify a suspected isolate as Xoo.

PHAGE ISOLATION FROM NATURALLY INFECTED SEEDS

General: This indirect method detects the pathogen by demonstrating the presence of its specific bacteriophage. With naturally infected seeds, one must first know the indicator bacterium (i.e., the corresponding Xoo strain sensitive to most of the Xoo-specific phages) to be added in the assay.

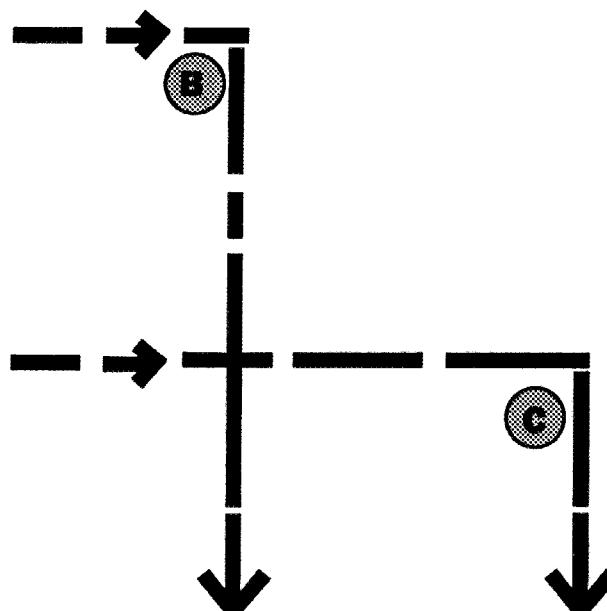
Procedure (see Fig. 7.14):

1. Macerate 100 seeds in 10 ml of sterile peptone sucrose broth (PSB).
2. Centrifuge this seed suspension at 10,000 rpm for 10 min. Keep the supernatant.
3. Add 1 ml of a full-grown broth culture of indicator bacteria to 1 ml of undiluted and to 1 ml of appropriate dilutions of the supernatant.
4. Add 3-4 ml autoclaved peptone sucrose agar (PSA) medium, cooled to 40 °C, to the sample. In a vortex mixer, shake samples carefully to achieve a homogeneous distribution. Pour samples into petri dishes and let them solidify.
5. Incubate the plates at 28 °C.
6. Check the plates the next day for plaque formation.

Results: If the sample contains phages specific to the Xoo indicator strain, there will be lysis as shown by plaque formation (Fig. 7.15). Hence, if the phage is present in the sample, so is the pathogen.

- Notes:**
1. It is advisable to confirm this indirect method of detecting Xoo by testing the specificity of the phages isolated from the infected seeds against Xoo, Xcola, *Erwinia herbicola* (a common contaminant in isolation of Xoo), and other yellow colonies isolated from the seeds.
 2. At IRRI, phages are most commonly isolated from ricefield water.

- A1. Macerate 100 seeds in 10 ml of sterile PSB.
- A2. Centrifuge at 10,000 rpm for 10 min.
- A3. Separate supernatant and pellet.
- A4. Remove 0.1 ml of supernatant and use it to determine the phage count in the original sample (steps B1-5)
- A5. Mix the remaining supernatant with the pellet, and add a known amount of species-specific phages (approximately 10^2 phages).
- A6. Immediately after adding the phages, centrifuge the sample again at 10,000 rpm for 10 min.
- A7. Separate supernatant and pellet.
- A8. Remove 0.1 ml of supernatant and use it to determine the phage count in the original sample plus the number of phages added in step A5 (steps C1-5).
- A9. In a vortex mixer, mix the remaining supernatant and pellet.
- A10. Incubate for 10 h at 28 °C. This allows the phages to infect Xoo bacteria present in the seed sample, resulting in multiplication of the phages.
- A11. Centrifuge at 10,000 rpm for 10 min.
- A12. Separate the supernatant and prepare serial dilutions up to 10^6 .
- A13. To 0.1 ml samples of each dilution, add 0.5 ml indicator bacteria that has been fully grown in PSB and 4 ml molten PSA that has been cooled to 10 °C.
- A14. Shake carefully and pour each into a petri dish. Incubate overnight at 28 °C.
- A15. Count the plaques. A significant increase indicates the presence of Xoo.



B1. While doing step A12, use the 0.1 ml of supernatant taken at step A4 and make dilutions up to 10^{-3} .

B2. Proceed as in step A13.

B3. Proceed as in step A14.

B4. Proceed as in step A15.

B5. Count plaques to estimate the number of phages in the original sample.

C1. While doing step A12, use the 0.1 ml of supernatant taken at step A8 and make dilutions up to 10^{-3} .

C2. Proceed as in step A13.

C3. Proceed as in step A14.

C4. Proceed as in step A15.

C5. Count plaques to ascertain the total number of phages which include those added in step A5, and the initial number of phages in the original sample.

7.14 Phage multiplication procedure.

PHAGE MULTIPLICATION

General: In the phage multiplication technique, a known amount of species-specific phage is added to the homogenized seed suspension for incubation at 28 °C for 10 h. A significant increase in phage number, determined by plaque count, indicates the presence of the bacterial cells in the seed sample.

Indeed, if the traced bacterium is present, the added specific phages will adsorb to and infect the bacterial cells, resulting in lysis of the bacteria through which a large amount of new phages are released. These, in turn, can infect other bacterial cells, and so on. The result is vast multiplication of phages, reflected through the number of lysed bacteria shown by clear zones on the medium (plaques).

Procedure: see Figure 7.14.

Results: A significant increase in phage number (plaque count at 10 h vs plaque count at 0 h) indicates the presence of Xoo in the seed sample.

Note: This method can provide an estimation of the amount of bacterial blight pathogens present in the seed sample, as long as the bacterial

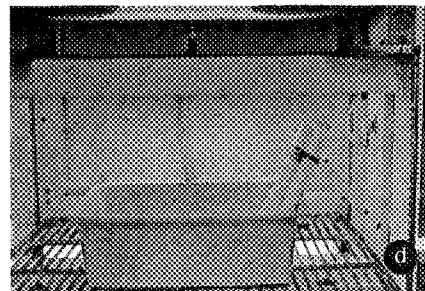
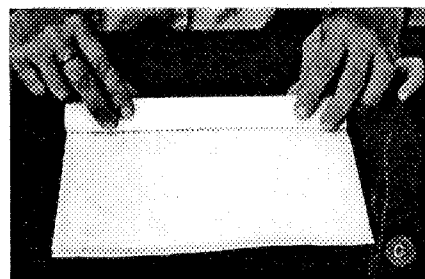
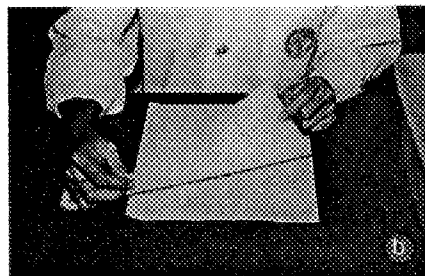
population is above the phage multiplication threshold. To estimate disease levels, also consider the average time the phage needs for one infection cycle and the average burst size (i.e., the average yield of virus particles per infected host cell). For further information, see *Bacteriophages* (Adams 1959).

Roll towel method to detect *Xanthomonas oryzae* pv. *oryzae* from rice seed (Singh and Rao 1977)

General: This test is similar to the growing-on test, but between wet paper towels. It is used to detect Xoo infection of seeds by examining the resulting seedlings. The method can be a routine procedure for seed health testing of suspected seed lots.

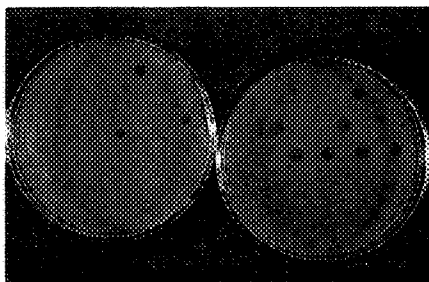
Procedure:

1. Soak two 45- x 28-cm paper towels in tap water.
2. Place 100 seeds, equally spaced in 10 replicates of 10 seeds on one of the wet paper towels (Fig. 7.16a). Use the other wet paper towel to cover the seeds (Fig. 7.16b).
3. Roll the towels (Fig. 7.16c) and close the ends with rubber bands. The rolled towels may be placed in a plastic bag to maintain humidity.
4. Place towels in an upright or inclined position in a plastic tray (Fig. 7.16d). Incubate the whole setup for 5-9 d at 28-30 °C under a 12-h light regime.
5. After 5-9 d, remove the rubber bands, unroll the towels, and examine the seedlings carefully for bacterial blight symptoms. Small pieces of coleoptile, leaf sheath, and leaf which show symptoms of water



7.16 Paper towel method. a. Plating 100 seeds on wet paper towel. b. Covering the seeds. c. Rolling the towels. d. Incubation position.

7.16 Plaque formation (clear zones result from lysis of the bacteria).



soaking (brown or yellow discoloration) must be examined microscopically for bacterial ooze.

6. Crush the pieces showing bacterial ooze in a little sterile physiological water (0.85% NaCl) in a petri dish.

7. Streak the bacteria onto PSA medium by dipping a wire loop in the suspension. Incubate the plates for 72-96 h at 28 °C.

Results: Examine the PSA plates for typical Xoo colonies. Pick up possible candidates and proceed with the purification requirements.

Using pure colonies, perform the pathogenicity test to confirm the identity of the isolates. ■